Effects of dietary supplementation with phytonutrients on vaccine-stimulated immunity against infection with *Eimeria tenella*[☆]

Sung Hyen Lee [a,1], Hyun S. Lillehoj [a,*], Seung I. Jang [a], Kyung Woo Lee [a], David Bravo [b], Erik P. Lillehoj [c]

[a] *Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service-U.S. Department of Agriculture, Beltsville, MD, USA*
[b] *Pancosma S.A., Geneva, Switzerland*
[c] *Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD, USA*

ARTICLE INFO

ABSTRACT

Two phytonutrient mixtures, VAC (carvacrol, cinnamaldehyde, and *Capsicum* oleoresin), and MC (*Capsicum* oleoresin and turmeric oleoresin), were evaluated for their effects on chicken immune responses following immunization with an *Eimeria* profilin protein. Chickens were fed with a non-supplemented diet, or with VAC- or MC-supplemented diets, immunized with profilin, and orally challenged with virulent oocysts of *Eimeria tenella*. Immunity against infection was evaluated by body weight, fecal oocyst shedding, profilin antibody levels, lymphocyte recall responses, cytokine expression, and lymphocyte subpopulations. Following immunization and infection, chickens fed the VAC- or MC-supplemented diets showed increased body weights, greater profilin antibody levels, and/or greater lymphocyte proliferation compared with non-supplemented controls. Prior to *Eimeria* infection, immunized chickens on the MC-supplemented diet showed reduced IFN-γ and IL-6 levels, but increased expression of TNFSF15, compared with non-supplemented controls. Post-infection levels of IFN-γ and IL-6 were increased, while IL-17F transcripts were decreased, with MC-supplementation. For VAC-supplemented diets, decreased IL-17F and TNFSF15 levels were observed only in infected chickens. Finally, immunized chickens fed the MC-supplemented diet exhibited increased MHC class II^{+}, CD4^{+}, CD8^{+}, TCR1+, or TCR2^{+} T cells compared with nonsupplemented controls. Animals on the VAC-containing diet only displayed an increase in K1^{+} macrophages. In conclusion, dietary supplementation with VAC or MC alters immune parameters following recombinant protein vaccination against avian coccidiosis.

Published by Elsevier B.V.

---

1. Introduction

Coccidiosis is an intestinal disease caused by multiple species of *Eimeria* (Protozoa, Apicomplexa). Avian coccidiosis is an economically important infection for the poultry industry worldwide that has traditionally been controlled with coccidiostatic drugs and antibiotic-based growth promoters (Lillehoj and Lillehoj, 2000; Lillehoj and Lee, 2007a,b; Lee et al., 2008a). Recent interest has focused on developing drug-free disease control strategies due, in part, to the emergence of drug-resistant parasites. In particular, an increasing reliance has been placed on live, attenuated *Eimeria* vaccines for control of avian coccidiosis (Williams et al., 1999; Williams and Catchpole, 2000). However, the potential development of additional diseases, such as necrotic enteritis due to the interaction of coccidia parasites with *Clostridium* bacteria, limits the utility of live parasite vaccines (Williams, 2002; Pogonka et al., 2003).

Eimeria recombinant protein vaccines, while less efficacious than coccidiostats and live vaccines, offer an alternative control strategy against avian coccidiosis (Lillehoj et al., 2000). For example, *Eimeria* profilin was identified in the merozoites of the parasite as an immunogenic protein which increased antigen-specific proliferation of chicken spleen lymphocytes and augmented cytokine production by splenic and intestinal lymphocytes (Lillehoj et al., 2000; Ding et al., 2004; Lillehoj and Okamura, 2003; Yarovinsky et al., 2005; Plattner et al., 2008; Lee et al., 2010b,d). Subcutaneous immunization of chickens with recombinant profilin expressed in *Escherichia coli* increased protective immunity to subsequent challenge infection by virulent *Eimeria acervulina* (Lillehoj et al., 2000, 2004). However, the effectiveness of profilin immunization was dose-dependent, such that only partial protection against challenge infection was generated at low vaccine doses ($\leq 100\,\mu g$).

A variety of approaches has been pursued to improve the effectiveness of recombinant protein vaccines, for example by coadministration of the vaccine with adjuvants or recombinant chicken cytokines, or by feeding plant-based phytonutrients (Min et al., 2001; Park et al., 2004; Lee et al., 2005, 2007a, 2008a,b, 2009c, 2010a; Kim et al., 2010; Jang et al., 2010). Previously, we showed that *in vitro* exposure of spleen cells to an extract of turmeric (*Curcuma longa*) increased lymphocyte proliferation compared with cells treated with vehicle alone (Lee et al., 2010a). Additionally, dietary supplementation of

chickens with carvacrol, cinnamaldehyde, or *Capsicum* oleoresin altered the *in vivo* expression of a family of innate immune response genes compared with chickens fed a non-supplemented diet (Kim et al., 2010). Therefore, we hypothesized that these phytonutrients would enhance vaccine-induced immunity against experimental *Eimeria* infection. To test this hypothesis, chickens were fed from hatch with normal diets, or with diets supplemented with carvacrol, cinnamaldehyde and *Capsicum* oleoresin (VAC) or with *Capsicum* plus turmeric oleoresins (MC), and the ability of recombinant profilin immunization to induce immunity against subsequent *Eimeria* challenge infection was compared between the experimental and control groups.

2. Materials and methods

2.1. Experimental animals and diets

A schematic diagram of the experimental protocol is shown in Fig. 1. One-day-old broiler chickens (Ross/Ross, Longenecker's Hatchery, Elizabethtown, PA) were housed in Petersime starter brooder units and randomly assigned to 4 groups (12 chickens/group). Chickens were continuously fed a standard diet or with the standard diet supplemented with VAC (5 mg/kg carvacrol, 3 mg/kg cinnamaldehyde, and 2 mg/kg *Capsicum* oleoresin) or MC (4 mg/kg turmeric oleoresin and 4 mg/kg *Capsicum* oleoresin) for 26 days after hatching. The concentration of supplements in the diet was based on our previous report (Lee et al., 2010a). All diets contained 24.2% protein, 54.0% carbohydrate, 3.7% vitamin and mineral mixture, 4.7% fat, and 2.4% fiber (USDA/FeedMill, Beltsville, MD). The birds were maintained in a temperature-controlled environment at 29 °C and fed with diets and water *ad libitum*. All experiments were approved by the USDA-Agricultural Research Service Institutional Animal Care and Use Committee (IACUC).

2.2. Recombinant profilin immunization and experimental infection

E. acervulina recombinant profilin was expressed in *E. coli* and purified as described (Jang et al., 2010). Chickens were mock immunized subcutaneously with PBS or vaccinated with 50 μg of recombinant profilin at 7 days post-hatch. At 17 days post-hatch, chickens were orally
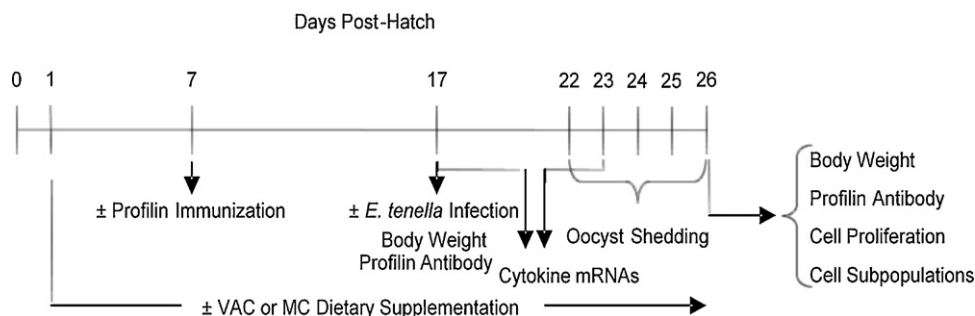
Days Post-Hatch

Fig. 1. Schematic illustration of the experimental protocol.

infected with 2.0×10^4 sporulated virulent oocysts of *E. tenella*, WLR-1 strain (Lee et al., 2008c, 2010b,d). The birds were kept in brooder pens in an *Eimeria*-free facility for 2 weeks prior to infection and transferred to hanging cages (2 birds/cage) at a separate location where they were infected and kept until the end of the experimental period.

2.3. Body weight and fecal oocyst shedding

Body weights were measured at 17 and 26 days post-hatch (0 and 9 days post-infection). Fecal oocysts were collected daily between 22 and 26 days post-hatch (5 and 9 days post-infection). Oocyst numbers were determined as described (Lee et al., 2010b,c,d) using a McMaster chamber according to the formula: total oocysts/bird = (oocyst count × dilution factor × [fecal sample volume/counting chamber volume])/2.

2.4. Anti-profilin antibody levels

Blood samples (4 chickens/group) were collected by cardiac puncture immediately following euthanasia at 17 and 26 days post-hatch, sera were obtained by low speed centrifugation, and used in an ELISA to measure profilin-specific antibody responses as described (Lee et al., 2007c,d, 2010b). Briefly, 96-well microtiter plates were coated overnight with $1.0 \,\mu$g/well of purified recombinant profilin. The plates were washed with phosphate-buffered saline containing 0.05% Tween (PBS-T) and blocked with PBS containing 1% BSA. Diluted sera (1:50) were added ($100 \,\mu$l/well), incubated with agitation for 2 h at room temperature, and washed with PBS-T. Bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgG and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma, St. Louis, MO). Optical density at 450 nm (OD_{450}) was measured with an automated microplate reader (Bio-Rad, Richmond, CA). All samples were analyzed in quadruplicate.

2.5. Spleen lymphocyte proliferation

At 26 days post-hatch, chickens (4/group) were euthanized by cervical dislocation, spleens were removed and placed in Petri dishes with 10 ml of Hanks' balanced salt solution (HBSS) supplemented with 100 U/ml penicillin and $100 \,\mu$g/ml streptomycin (Sigma). Cell suspensions were prepared by gently flushing through a cell strainer and lymphocytes were purified by density gradient centrifugation through Histopaque-1077 (Sigma). The cells were adjusted to 5.0×10^6 cells/ml in RPMI medium containing 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT), 100 U/ml penicillin, and $100 \,\mu$g/ml streptomycin and incubated with medium alone or with $20 \,\mu$g/ml of profilin in 96-well plates in a humidified incubator (Forma, Marietta, OH) at $41 \,^\circ$C and 5% CO_2 for 24 h. Cell proliferation was measured using WST-8 (Dojindo Molecular Technologies, Gaithersburg, MD) at OD_{450} using a microplate spectrophotometer (Bio-Rad) as described (Lee et al., 2010a,b). WST-8 is reduced by dehydrogenases in cells to give a yellow-colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Lymphoproliferation was expressed as stimulation index equal to the mean OD_{450} value of the profilin-stimulated group divided by the mean OD_{450} value of the medium-only stimulated group.

2.6. Intestinal cytokine mRNA levels

Cecal tonsils were obtained from non-infected and infected chickens at 17 and 23 days post-hatch (6 days post-*Eimeria* infection) (4/group), respectively, the tissues were cut longitudinally, and washed three times with ice-cold HBSS containing 100 U/ml of penicillin and $100 \,\mu$g/ml of streptomycin as described (Lee et al., 2010a). The mucosal layer was carefully scraped away using a surgical scalpel and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I in $1.0 \,\mu$l of $10\times$ reaction buffer (Sigma) for 15 min at room temperature, $1.0 \,\mu$l of stop solution was added to inactivate DNase I, and the mixture was heated at $70 \,^\circ$C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide primers for chicken interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-17F, and tumor necrosis factor superfamily 15 (TNFSF15) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA using the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA. Levels

Table 1
Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines.

RNA target	Primer sequences	PCR product size (bp)	Accession No.
GAPDH	F: 5′-GGTGGTGCTAAGCGTGTTAT-3′	264	K01458
	R: 5′-ACCTCTGTCATCTCTCCACA-3′		
IFN-γ	F: 5′-AGCTGACGGTGGACCTATTATT-3′	259	Y07922
	R: 5′-GGCTTTGCGCTGGATTC-3′		
IL-6	F: 5′-CAAGGTGACGGAGGAGGAC-3′	254	AJ309540
	R: 5′-TGGCGAGGAGGGATTTCT-3′		
IL-17F	F: 5′-CTCCGATCCCTTATTCTCCTC-3′	292	AJ493595
	R: 5′-AAGCGGTTGTGGTCCTCAT-3′		
TNFSF15	F: 5′-CCTGAGTATTCCAGCAACGCA-3′	292	NM010245578
	R: 5′-ATCCACCAGCTTGATGTCACTAAC-3′		

of individual transcripts were then normalized to those of GAPDH and analyzed by the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression prior to calculating means and the SEM for the references and individual targets, followed by determination of the mean normalized expression using the Q-gene program (Lee et al., 2008b).

2.7. Flow cytometric analysis

At 26 days post-hatch, chickens (4/group) were euthanized by cervical dislocation and bled by cardiac puncture using a heparinized syringe. Single cell suspensions of peripheral blood lymphocytes (PBL) were prepared as described (Lee et al., 2007a,b), resuspended in HBSS without phenol red supplemented with 3% fetal bovine serum and 0.01% sodium azide (FCA buffer), and adjusted to 1.0×10^7 cells/ml in FCA buffer. The cells were incubated for 30 min on ice with mouse monoclonal antibodies (mAbs) specific for chicken major histocompatibility complex (MHC) class II, CD4, CD8, K1, T cell receptor 1 (TCR1), or TCR2 surface proteins (Lillehoj and Chai, 1988; Lee et al., 2008c). An antibody against chicken MHC class I antigens, C6B12, was used as a positive control and HB2, an antibody specific for human T cells (American Type Culture Collection, Manassas, VA), was used as a negative control. Following incubation, the cells were washed three times with FCA buffer and incubated with 100 μl of fluorescein isothiocyanate-labeled goat anti-mouse IgG secondary antibody (Sigma) for 45 min on ice. The cells were washed three times with FCA buffer, and fluorescence was then analyzed with 1.0×10^4 viable cells using a FACSCalibur (BD, Boston, MA).

2.8. Statistical analyses

Statistical analyses were performed using SPSS software (SPSS 15.0 for Windows, Chicago, IL) and all data were expressed as the mean ± SEM values. Comparisons of the mean values were performed by one-way analysis of variance, followed by the Duncan's multiple range test, and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of dietary supplementation with VAC or MC on body weight and fecal oocyst shedding

Our previous studies demonstrated that immunization of chickens with a suboptimal dose (≤ 100 μg/chicken) of recombinant profilin failed to provide complete protection against subsequent challenge infection with live parasites (Lillehoj et al., 2000, 2004). Therefore, we selected a 50 μg dose to evaluate the ability of the VAC and MC phytonutrients to augment the efficacy of profilin vaccination. Initially, we determined that neither phytonutrient mixtures exhibited gross toxic effects when used at the denoted
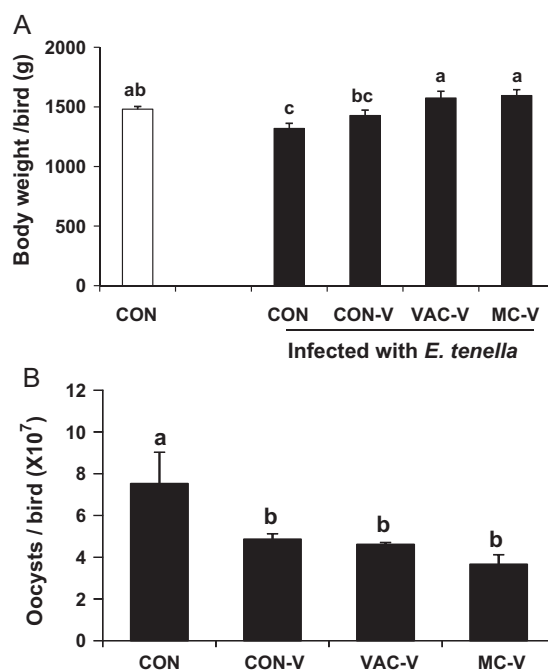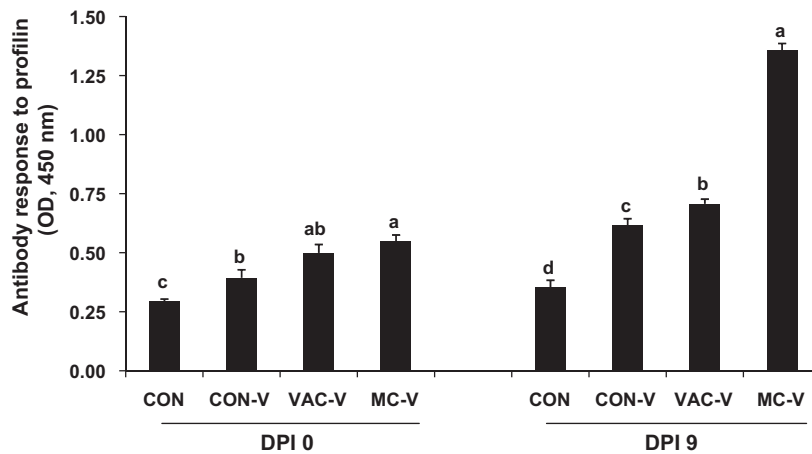

Fig. 2. Effect of dietary supplementation with VAC or MC on body weight and fecal oocyst shedding. One-day-old chickens were fed a standard diet alone or a standard diet supplemented with VAC or MC. At 7 days post-hatch, chickens were unimmunized (open bar) or subcutaneously immunized with PBS (CON) or 50 μg of recombinant profilin/bird (CON-V, VAC-V, MC-V). At 17 days post-hatch, chickens were uninfected (open bar) or orally infected with 2.0×10^4 sporulated oocysts of *E. tenella* (closed bars). (A) Body weights were measured at 26 days post-hatch. (B) Total fecal oocysts were counted at 22 and 26 days post-hatch. Each bar represents the mean value ± SEM ($n = 12$). Bars not sharing the same letter are significantly different ($P < 0.05$) according to the Duncan's multiple range test.

concentrations. Further, continuous feeding with VAC- or MC-supplemented diets during the first 17 days post-hatch and prior to *E. tenella* infection did not affect body weights compared with the non-supplemented diet group (data not shown). Following immunization with recombinant profilin and *E. tenella* infection, chickens fed the VAC- or MC-supplemented diets exhibited increased body weights by 8% and 21%, respectively, compared with immunized and infected animals given the non-supplemented diet (Fig. 2A). Indeed, there was no significant difference in body weights between non-infected controls and infected chickens fed the VAC- or MC-supplemented diets. As shown in Fig. 2B, fecal oocyst shedding was not affected in profilin-immunized and *E. tenella*-infected chickens by dietary supplementation with VAC or MC, compared with immunized and infected animals given the non-supplemented diet.

3.2. Effect of dietary supplementation with VAC or MC on profilin serum antibody levels

Anti-profilin serum antibody levels increased in immunized chickens and were significantly higher in the chickens fed the VAC- or MC-supplemented diets, com-
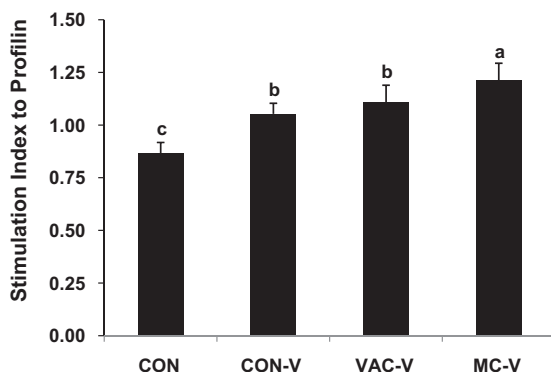
Fig. 3. Effect of dietary supplementation with VAC or MC on profilin serum antibody levels. One-day-old chickens were fed a standard diet alone (CON, CON-V) or a standard diet supplemented with VAC or MC. At 7 days post-hatch, chickens were subcutaneously immunized with PBS (CON) or 50 μg of recombinant profilin/bird (CON-V, VAC-V, MC-V). At 17 days post-hatch, chickens were orally infected with 2.0×10^4 sporulated oocysts of E. tenella. At 17 (0 DPI) and 26 days post-hatch (9 DPI), serum anti-profilin antibody levels were measured by ELISA. Each bar represents the mean value $\pm$ SEM ($n = 4$). Bars not sharing the same letter are significantly different according to the Duncan's multiple range test ($P < 0.05$).

pared with immunized and infected animals given the non-supplemented diet (Fig. 3). MC was superior to VAC in augmenting profilin antibody levels.

3.3. Effect of dietary supplementation with VAC or MC on spleen lymphocyte proliferation

Profilin-driven spleen lymphocyte proliferation was increased in the profilin-immunized and parasite-infected group given the MC-supplemented diet compared with immunized and infected animals fed the non-supplemented diet (Fig. 4). VAC supplementation had no

Fig. 4. Effect of dietary supplementation with VAC or MC on spleen lymphocyte proliferation. One-day-old chickens were fed a standard diet alone (CON, CON-V) or a standard diet supplemented with VAC or MC. At 7 days post-hatch, chickens were subcutaneously immunized with PBS (CON) or 50 μg of recombinant profilin/bird (CON-V, VAC-V, MC-V). At 17 days post-hatch, chickens were orally infected with 2.0×10^4 sporulated oocysts of E. tenella. At 26 days post-hatch, spleen lymphocytes were treated with 20 μg/ml of profilin and cell proliferation was measured. Each bar represents the mean $\pm$ SEM value ($n = 4$). Bars not sharing the same letter are significantly different according to the Duncan's multiple range test ($P < 0.05$).

effect on the splenocyte proliferative response compared with the non-supplemented controls.
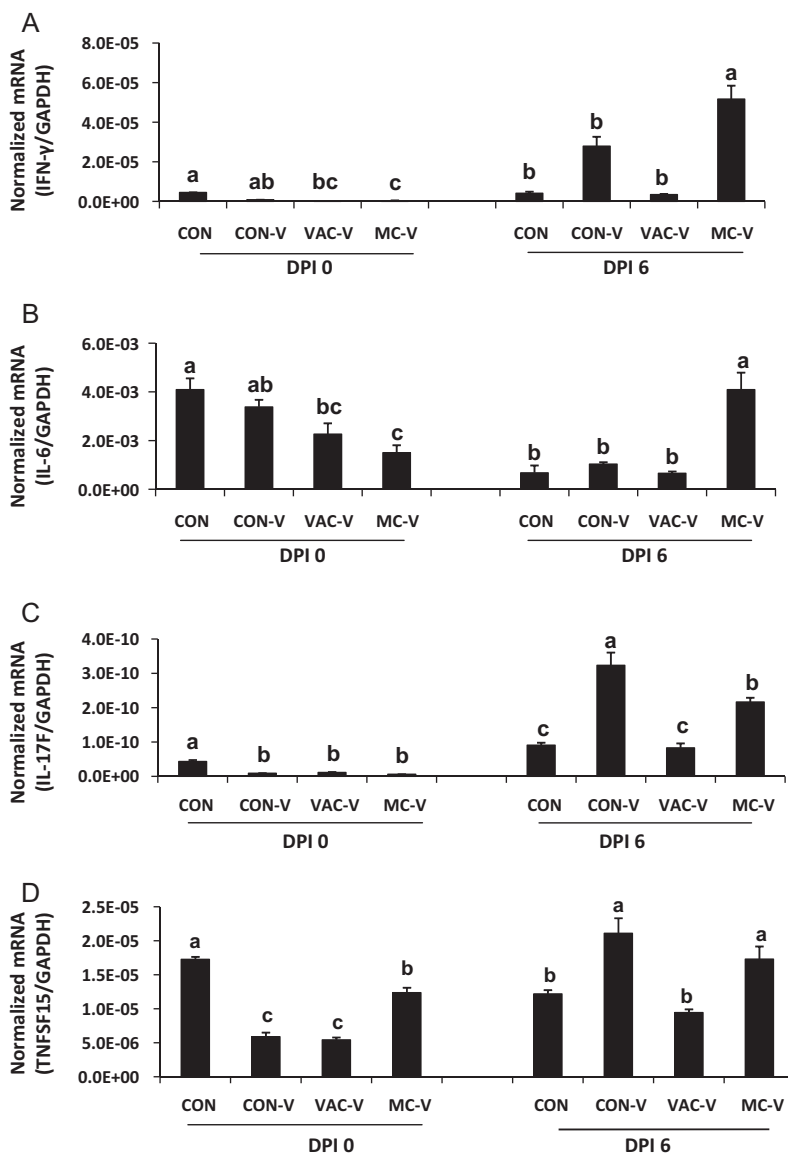
3.4. Effect of dietary supplementation with VAC or MC on intestinal cytokine mRNA levels

Fig. 5 illustrates the effect of dietary supplementation with VAC or MC on the levels of mRNAs for IFN-γ, IL-6, IL-17F, and TNFSF15 in intestinal tissues of profilin-immunized chickens either prior to Eimeria infection (17 days post-hatch) or at 6 days following infection (23 days post-hatch). Profilin-immunized chickens fed the MC-supplemented diet displayed reduced levels of transcripts for IFN-γ and IL-6, but increased TNFSF15 transcripts, prior to Eimeria infection, compared with non-supplemented and immunized controls. By contrast, the levels of IFN-γ and IL-6 transcripts were increased, while IL-17F transcripts were decreased in parasite-infected animals given the MC-supplemented diet compared with non-supplemented and immunized controls. For VAC-supplemented diets, decreased levels of transcripts for IL-17F and TNFSF15 compared with non-supplemented and immunized controls were observed only in infected chickens.

3.5. Effect of dietary supplementation with VAC or MC on PBL subpopulations

Chicken lymphocytes play a critical role in the host immune response to Eimeria infection. Therefore, we next assessed the changes in PBL subpopulations in profilin-immunized and parasite-infected chickens given the VAC, MC or control diets. Animals fed the MC-supplemented diet exhibited increased percentages of MHC class II-, CD4-, CD8-, TCR1-, and TCR2-positive cells compared with non-supplemented and immunized controls, while animals

Fig. 5. Effect of dietary supplementation with VAC or MC intestinal cytokine mRNA levels. One-day-old chickens were fed a standard diet alone (CON, CON-V) or a standard diet supplemented with VAC or MC. At 7 days post-hatch, chickens were subcutaneously immunized with PBS (CON) or 50 μg of recombinant profilin/bird (CON-V, VAC-V, MC-V). At 17 days post-hatch, chickens were orally infected with 2.0×10^4 sporulated oocysts of *E. tenella*. At 17 (0 DPI) and 23 days post-hatch (6 DPI), the levels of intestinal mRNAs encoding IFN-γ, IL-6, IL-17F, and TNFSF15 were measured by quantitative RT-PCR. Each bar represents the mean ± SEM value (*n* = 4). Bars not sharing the same letter are significantly different according to the Duncan's multiple range test (*P* < 0.05).

given the VAC diet only displayed an increased fraction of K1-staining macrophages (Table 2).

4. Discussion

This study demonstrated that dietary supplementation of broiler chickens with phytonutrient mixtures, carvacrol, cinnamaldehyde, *Capsicum* and/or turmeric enhanced immune responses that were induced by vaccination with an *Eimeria* recombinant profilin protein. Birds on the VAC- or MC-supplemented diets exhibited higher body weights, compared with the non-supplemented control group.

Analysis of a series of parameters of innate and adaptive immunity revealed a more robust post-immunization response stimulated by the MC-supplemented diet compared with the VAC diet. Thus, immunized chickens given the MC diet displayed increased serum anti-profilin antibody levels, greater profilin-induced lymphocyte proliferation, altered levels of mRNAs encoding IFN-γ, IL-6, IL-17F, and TNFSF15 in the intestine, and increased percentages of MHC class II-, CD4-, CD8-, TCR1-, and TCR2-staining PBLs, compared not only with non-supplemented controls, but also with the VAC diet group. Given that both phytonutrient mixtures contained similar amounts

Table 2
Effect of dietary supplementation with VAC or MC on PBL subpopulations.

mAb/%	CON	CON-V	VAC-V	MC-V
MHC class II	10.6 ± 1.3[c]	21.3 ± 1.8[b]	18.6 ± 1.1[b]	54.7 ± 4.1[a]
CD4	7.9 ± 1.0[c]	17.3 ± 1.6[b]	16.2 ± 1.1[b]	32.8 ± 1.8[a]
CD8	11.4 ± 1.4[c]	23.3 ± 1.8[b]	23.3 ± 1.9[b]	44.2 ± 3.5[a]
K1	1.9 ± 0.2[b]	2.1 ± 0.1[b]	3.9 ± 0.2[a]	1.8 ± 0.1[b]
TCR1	2.1 ± 0.3[b]	3.6 ± 0.3[b]	2.8 ± 0.1[b]	9.0 ± 0.6[a]
TCR2	6.9 ± 0.6[b]	9.1 ± 0.5[b]	13.4 ± 0.8[b]	30.0 ± 1.6[a]

One-day-old broiler chickens were fed a standard diet alone (CON, CON-V) or a standard diet supplemented with VAC (VAC-V) or MC (MC-V). At 7 days post-hatch, chickens were immunized with 50 μg of recombinant profilin. At 17 days post-hatch, chickens were infected with 2.0×10^4 sporulated oocysts of *E. tenella*. At 26 days post-hatch, PBLs were analyzed by flow cytometric staining with the indicated monoclonal antibody (mAb). Each value represents the mean ± SEM ($n = 4$). Values not sharing the same letter within a row are significantly different ($P < 0.05$) according to the Duncan's multiple range test.

of *Capsicum*, the differences between MC and VAC diets likely represents the presence of turmeric in the former.

Several earlier studies have investigated the beneficial effects of dietary phytonutrients in mammals and chickens. In mammals, extracts from plants of the genus *Curcuma*, including *C. longa* (turmeric), exhibited anti-oxidant and anti-inflammatory properties (Policegoudra et al., 2007; Sodsai et al., 2007; Mannangatti and Narayanasamy, 2008). In chickens, dietary phytonutrients have improved the digestibility of feed, reduced intestinal pathogenic bacterial load, and counteracted *Eimeria* parasite-induced body weight loss (Jamroz et al., 2003, 2005; Lee et al., 2010a,c). Our recent report showed increased levels of mRNAs encoding IL-6 and IFN-γ in chicken macrophages treated *in vitro* with turmeric extract, as well as increased proliferation of chicken spleen cells treated with the extract, compared with non-treated controls (Lee et al., 2010a). We also have demonstrated that dietary *Capsicum* oleoresin reduced the expression of intestinal proinflammatory cytokines, improved body weight, and reduced parasite fecundity in chickens infected with *E. acervulina*, compared with animals provided with a non-supplemented diet (Lee et al., 2010c). Carvacrol is one of the most common components of essential oils and possesses a wide range of anti-microbial activities (Casewell et al., 2003; Ben Arfa et al., 2006; Chami et al., 2005; Veldhuizen et al., 2006). Carvacrol has been approved as a safe food additive in the US and Europe (Center for Food Safety and Applied Nutrition, 2006). Cinnamaldehyde, the other phytonutrient used in this study, has been shown to exhibit anti-fungal, anti-pyretic, anti-oxidant, anti-microbial, and larvicidal activities, in addition to modulating T cell differentiation (Lin et al., 2003; Cheng et al., 2004; Kim et al., 2004; Koh et al., 1998).

We observed that markers of both humoral immunity (profilin-specific antibody) and cell-mediated immunity (T cell proliferation, lymphocyte subpopulations) were enhanced by the MC diet. Cell-mediated immunity has been shown to play a major role in protection against avian coccidiosis (Lillehoj and Ruff, 1987; Lee et al., 2007a,b, 2008c, 2010a,c). Antibody-mediated immunity also appears to play a role in passive immune protection against this disease (Lillehoj et al., 2004; Lee et al.,

2009a,b). The increased level of antibodies against parasite antigens of survival importance, such as profilin, likely enhances local protection against coccidiosis. Current studies in our laboratory are directed at measuring anti-profilin antibody levels in intestinal secretions of chickens given phytonutrient-supplemented diets.

The host cell-immune response to *Eimeria* is initiated and amplified through the production of multiple proinflammatory cytokines in the intestine, including IFN-γ, IL-6, IL-17F, and TNFSF15 (Lillehoj et al., 2001, 2007; Hong et al., 2006a,b; Lee et al., 2008b). Cytokines are critical for local immunoregulation and protection against intracellular parasites in general and coccidia in particular. IFN-γ is a common marker of cellular immunity and has been shown to be associated with protective immune responses to avian coccidiosis (Min et al., 2003; Lillehoj et al., 2004; Lee et al., 2008c). For example, administration of purified recombinant IFN-γ to chickens increased resistance against experimental coccidiosis, significantly reduced intracellular development of *Eimeria* parasites, and exerted an immune adjuvant effect when given simultaneously in a DNA vaccine (Lillehoj and Choi, 1998; Min et al., 2001). IL-6, a cytokine produced by T cells and macrophages, acts as both a pro-inflammatory and an anti-inflammatory cytokine, depending upon the experimental context (Kanegane and Tosato, 1996; Choi and Lillehoj, 2000). IL-6 and IFN-γ transcript levels in the gut were significantly up-regulated following *E. tenella* infection, with maximum responses at 6 days post-infection (Hong et al., 2006b), and our current results indicate that MC supplementation further increases the expression of these two cytokines at this time. IL-17 was originally described as a cytokine secreted exclusively by activated memory T cells that induced fibroblasts to secrete other cytokines involved in proinflammatory or hematopoietic processes, such as IL-6, IL-8 and granulocyte-colony stimulating factor (Yao et al., 1995). Subsequently, a number of homologous proteins comprising an IL-17 family were identified, including IL-17A (original IL-17), -17B, -17C, -17D, -17E, and -17F (Pappu et al., 2008). IL-17F is a cytokine secreted exclusively by activated memory T cells that induces fibroblasts to secrete additional soluble mediators of inflammation. Following primary infection with *E. acervulina* or *E. maxima,* but not *E. tenella*, intestinal IL-17F expression at the mRNA level was highly up-regulated (Lee et al., 2010b; Hong et al., 2006a,b). While the present study corroborates the inability of *E. tenella* to increase IL-17F expression, the responsible molecular and cellular mechanisms are unknown. TNFSF and TNFSF receptor proteins are essential for the differentiation, proliferation, and function of immune cells (Collette et al., 2003). In chickens, recombinant TNFSF15 exhibited a dose-dependent cytotoxic effect on HTC and LSCC-RP9 tumor cells (Park et al., 2007). In addition, a previous study provided evidence that chicken TNFSF15 decreased feed intake, increased rectal temperature, and exerted a direct cytotoxic effect against murine fibroblasts or primary chicken cell cultures (Takimoto et al., 2005).

Analysis of lymphocyte subpopulation expressing various cell surface markers provides an additional line of support for the beneficial effects of dietary phytonutrients

on profilin-stimulated avian immunity against coccidiosis. In particular, we observed that major T lymphocyte subsets (CD4^{+}, CD8^{+}, TCR1^{+}, and TCR2^{+}) were increased in profilin-immunized chickens fed the MC-supplemented diet and infected with *E. tenella*. CD4^{+} T cells are the main IFN-γ-producing cells in response to antigen challenge (McSorley et al., 2000). In chickens, CD8^{+} cells play an important role as direct effector cells in mediating protective immunity during avian coccidiosis (Lillehoj and Trout, 1996). In murine experimental models of apicomplexan parasite infections, both CD4^{+} and CD8^{+} T cells serve as effector cells and local producers of cytokines that regulate host responses (Sher and Coffman, 1992). TCR1^{+} lymphocytes that express the γ/δ polypeptide receptor for antigen recognition are capable of mediating specific cellular immune functions without antigen processing, and can directly recognize invading pathogens or damaged cells (Schild et al., 1994; Wallace et al., 1995). These cells are also capable of modulating the secretion of immunoregulatory cytokines as well as other soluble factors that influence epithelial cell growth and repair at mucosal surfaces (Boismenu et al., 1996). Unlike mammals, TCR1^{+} cells are a major circulating T cell subset in chickens and, in avian coccidiosis, are involved in cytotoxic effecter mechanisms that are considered vital for disease control (Lee et al., 2008c; Trout and Lillehoj, 1996; Lillehoj and Ruff, 1987). TCR2^{+} (α/β) cells secrete important immunoregulatory cytokines and have been suggested to play a critical role in mediating a cytotoxic effect against intracellular parasites such as *Eimeria* (Trout and Lillehoj, 1996).

In conclusion, the results of this investigation demonstrate that profilin-immunized chickens fed the VAC- or MC-supplemented diets and infected with *E. tenella* had increased body weights, greater anti-profilin antibody levels, heightened profilin-induced lymphocyte proliferation, altered intestinal cytokine transcript levels, and augmented percentages of PBL subpopulations, compared with immunized and infected animals provided with a non-supplemented diet. Although the underlying mechanisms that are responsible for the dietary immune enhancing effects remain to be determined, these results provide the foundation for further studies to identify a safe and effective alternative to prophylactic medication for control of avian coccidiosis in commercial poultry production facilities.

Acknowledgments

References

Ben Arfa, A., Combes, S., Preziosi-Belloy, L., Gontard, N., Chalier, P., 2006. Antimicrobial activity of carvacrol related to its chemical structure. Lett. Appl. Microbiol. 43, 149–154.

Boismenu, R., Feng, L., Xia, Y.Y., Chang, J.C., Havran, W.L., 1996. Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. J. Immunol. 157, 985–992.

Casewell, M., Friis, C., Marco, E., McMullin, P., Ian Phillips, I., 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52, 159–161.

Center for Food Safety and Applied Nutrition, 2006. EAFUS: A Food Additive Database. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC.

Chami, N., Bennis, S., Chami, F., Aboussekhra, A., Remmal, A., 2005. Study of anticandidal activity of carvacrol and eugenol *in vitro* and *in vivo*. Oral Microbiol. Immunol. 20, 106–111.

Cheng, S.S., Liu, J.Y., Tsai, K.H., Chen, W.J., Chang, S.T., 2004. Chemical composition and mosquito larvicidal activity of essential oils from leaves of different *Cinnamomum osmophloeum* provenances. J. Agric. Food Chem. 52, 4395–4400.

Choi, K.D., Lillehoj, H.S., 2000. Role of chicken IL-2 on γδ T-cells and *Eimeria acervulina*-induced changes in intestinal IL-2 mRNA expression and γδ T-cells. Vet. Immunol. Immunopathol. 73, 309–321.

Collette, Y., Gilles, A., Pontarotti, P., Olive, D., 2003. A co-evolution perspective of the TNFSF and TNFRSF families in the immune system. Trends Immunol. 24, 387–394.

Ding, X., Lillehoj, H.S., Quiroz, M.A., Bevensee, E., Lillehoj, E.P., 2004. Protective immunity against *Eimeria acervulina* following *in ovo* immunization with a recombinant subunit vaccine and cytokine genes. Infect. Immun. 72, 6939–6944.

Hong, Y.H., Lillehoj, H.S., Lillehoj, E.P., Lee, S.H., 2006a. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet. Immunol. Immunopathol. 114, 259–272.

Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R.A., Lillehoj, E.P., 2006b. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114, 209–223.

Jamroz, D., Wertelecki, T., Orda, J., Wiliczkiewicz, A., Skorupinska, J., Kamel, C., 2003. The influence of phytogenic extract on performance, nutrients digestibility, carcass characteristic and gut microbial status in broiler chickens. J. Anim. Feed Sci. 12, 583–596.

Jamroz, D., Wiliczkiewicz, A., Wertelecki, T., Orda, J., Skorupinska, J., 2005. Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. Brit. Poultry Sci. 46, 485–493.

Jang, S.I., Lillehoj, H.S., Lee, S.H., Lee, K.W., Park, M.S., Bauchan, G.R., Lillehoj, E.P., Bertrand, F., Dupuis, L., Deville, S., 2010. Immunoenhancing effects of Montanide[TM] ISA oil-based adjuvants on recombinant coccidia antigen vaccination against Eimeria acervulina infection. Vet. Parasitol. 172, 221–228.

Kanegane, H., Tosato, G., 1996. Activation of naive and memory T cells by interleukin-15. Blood 88, 230–235.

Kim, H.O., Park, S.W., Park, H.D., 2004. Inactivation of *Escherichia coli* O157, H7 by cinnamaldehyde purified from Cinnamomum cassia shoot. Food Microbiol. 21, 105–110.

Kim, D.K., Lillehoj, H.S., Lee, H.S., Jang, S.I., Bravo, D., 2010. High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or *Capsicum* oleoresin. Poultry Sci. 89, 68–81.

Koh, W.S., Yoon, S.Y., Kwon, B.M., Jeong, T.C., Nam, K.S., Han, M.Y., 1998. Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. Int. J. Immunopharmacol. 20, 643–660.

Lee, S.H., Park, J.B., Park, H.J., Park, Y.J., Sin, J.I., 2005. Biological properties of different types and parts of the dandelions: comparisons of antioxidative, immune cell proliferative and tumor cell growth inhibitory activities. J. Food Sci. Nutr. 10, 172–178.

Lee, S.H., Lillehoj, H.S., Chun, H.K., Tuo, W., Park, H.J., Cho, S.M., Lee, Y.M., Lillehoj, E.P., 2007a. *In vitro* treatment of chicken peripheral blood lymphocytes, macrophages, and tumor cells with extracts of Korean medicinal plants. Nutr. Res. 27, 362–366.

Lee, S.H., Lillehoj, H.S., Park, D.W., Hong, Y.H., Cho, S.M., Chun, H.K., Park, H.J., 2007b. Immunomodulatory effects of dietary safflower leaf in chickens. Kor. J. Commun. Living Sci. 18, 715–724.

Lee, S.H., Lillehoj, H.S., Dalloul, R.A., Park, D.W., Hong, Y.H., Lin, J.J., 2007c. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. Poultry Sci. 86, 63–66.

Lee, S.H., Lillehoj, H.S., Park, D.W., Hong, Y.H., Lin, J.J., 2007d. Effects of *Pediococcus* and *Saccharomyces*-based probiotic (MitoMax®) on coccidiosis broiler chickens. Comp. Immunol. Microbiol. Infect. Dis. 30, 261–268.

Lee, S.H., Lillehoj, H.S., Lillehoj, E.P., Chun, H.K., Park, H.J., Cho, S.M., 2008a. *In vitro* effects of methanol extracts of Korean medicinal fruits (Persimmon, Raspberry, Tomato) on chicken lymphocytes, macrophages, and tumor cells. J. Poultry Sci. 46, 149–154.

Lee, S.H., Lillehoj, H.S., Hong, Y.H., Cho, S.M., Park, D.W., Park, H.J., Chun, H.K., 2008b. Protective effects of dietary safflower (Carthamus tinctorius) on experimental coccidiosis. J. Poultry Sci. 46, 155–162.

Lee, S.H., Lillehoj, H.S., Lillehoj, E.P., Cho, S.M., Park, D.W., Hong, Y.H., Chun, H.K., Park, H.J., 2008c. Immunomodulatory properties of dietary plum on coccidiosis. Comp. Immunol. Microbiol. Infect. Dis. 31, 389–402.

Lee, S.H., Lillehoj, H.S., Park, D.W., Jang, S.I., Morales, A., Garcia, D., Lucio, E., Larios, R., Victoria, G., Marrufo, D., Lillehoj, E.P., 2009a. Induction of passive immunity in broiler chickens against Eimeria acervulina by hyperimmune egg yolk immunoglobulin Y. Poultry Sci. 88, 562–566.

Lee, S.H., Lillehoj, H.S., Park, D.W., Jang, S.I., Morales, A., Garcia, D., Lucio, E., Larios, R., Victoria, G., Marrufo, D., Lillehoj, E.P., 2009b. Protective effect of hyperimmune egg yolk IgY antibodies against Eimeria tenella and Eimeria maxima infections. Vet. Parasitol. 163, 123–126.

Lee, S.H., Lillehoj, H.S., Cho, S.M., Chun, H.K., Park, H.J., Lim, C.I., Lillehoj, E.P., 2009c. Immunostimulatory effects of oriental plum (Prunus salicina Lindl.). Comp. Immunol. Microbiol. Infect. Dis. 32, 407–417.

Lee, S.H., Lillehoj, H.S., Hong, Y.H., Jang, S.I., Lillehoj, E.P., Ionescu, C., Mazuranok, L., Bravo, D., 2010a. In vitro effects of plant and mushroom extracts on immunological function of chicken lymphocytes and macrophages. Brit. J. Polit. Sci. 51, 213–221.

Lee, S.H., Lillehoj, H.S., Jang, S.I., Lee, K.W., Yancey, R.J., Dominowski, P., 2010b. Effects of novel adjuvant complex/Eimeria profilin vaccine on intestinal host immune responses against live E. acervulina challenge infection. Vaccine 28, 6498–6504.

Lee, S.H., Lillehoj, H.S., Jang, S.I., Kim, D.K., Ionescu, C., Bravo, D., 2010c. Effect of dietary Curcuma, Capsicum, and Lentinus, on enhancing local immunity against Eimeria acervulina infection. J. Poultry Sci. 47, 89–95.

Lee, S.H., Lillehoj, H.S., Jang, S.I., Lee, K.W., Yancey, R.J., Dominowski, P., 2010d. Embryo vaccination of chickens using a novel adjuvant formulation stimulates protective immunity against Eimeria maxima infection. Vaccine 28, 7774–7778.

Lillehoj, H.S., Ruff, M.D., 1987. Comparison of disease susceptibility and subclass-specific antibody response in SC and FP chickens experimentally inoculated with Eimeria tenella, E. acervulina, or E. maxima. Avian Dis. 31, 112–119.

Lillehoj, H.S., Chai, J.Y., 1988. Comparative natural killer cell activities of thymic, bursal, splenic and intestinal intraepithelial lymphocytes of chickens. Dev. Comp. Immunol. 12, 629–643.

Lillehoj, H.S., Trout, J.M., 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites. Clin. Microbiol. Rev. 9, 349–360.

Lillehoj, H.S., Choi, K.D., 1998. Recombinant chicken interferon-gamma-mediated inhibition of Eimeria tenella development in vitro and reduction of oocyst production and body weight loss following Eimeria acervulina challenge infection. Avian Dis. 42, 307–314.

Lillehoj, H.S., Lillehoj, E.P., 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Dis. 44, 408–425.

Lillehoj, H.S., Choi, K.D., Jenkins, M.C., Vakharia, V.N., Song, K.D., Han, J.Y., Lillehoj, E.P., 2000. A recombinant Eimeria protein inducing interferon-gamma production: comparison of different gene expression systems and immunization strategies for vaccination against coccidiosis. Avian Dis. 44, 379–389.

Lillehoj, H.S., Min, W., Choi, K.D., Babu, U.S., Burnside, J., Miyamoto, T., Rosenthal, B.M., Lillehoj, E.P., 2001. Molecular, cellular, and functional characterization of chicken cytokines homologous to mammalian IL-15 and IL-2. Vet. Immunol. Immunopathol. 82, 229–244.

Lillehoj, H.S., Okamura, M., 2003. Host immunity and vaccine development to coccidia and Salmonella infections in chickens. J. Poultry Sci. 40, 151–193.

Lillehoj, H.S., Min, W., Dalloul, R.A., 2004. Recent progress on the cytokine regulation of intestinal immune response to Eimeria. Poultry Sci. 83, 611–623.

Lillehoj, H.S., Kim, C.H., Keeler Jr., C.L., Zhang, S., 2007. Immunogenomic approaches to study host immunity to enteric pathogens. Poultry Sci. 86, 1491–1500.

Lillehoj, H.S., Lee, S.H., 2007a. Probiotics as an alternative control strategy against avian coccidiosis. Feedinfo News Service Scientific Reviews. http://www.feedinfo.com.

Lillehoj, H.S., Lee, S.H., 2007b. Dietary modulation of intestinal innate immunity using plant-derived phytochemicals. Feedinfo News Service Scientific Reviews. http://www.feedinfo.com.

Lin, C.C., Wu, S.J., Chang, C.H., Ng, L.T., 2003. Antioxidant activity of Cinnamomum cassia. Phytother. Res. 17, 726–730.

Mannangatti, K., Narayanasamy, M., 2008. Antifungal protein from a medicinal plant, Curcuma caesia Roxb. J. Biotechnol. 136, S90.

McSorley, S.J., Cookson, B.T., Jenkins, M.K., 2000. Characterization of CD4+ T cell responses during natural infection with Salmonella typhimurium. J. Immunol. 164, 986–993.

Min, W., Lillehoj, H.S., Burnside, J., Weining, K.C., Staeheli, P., Zhu, J.J., 2001. Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4 and lymphotactin on DNA vaccination against Eimeria acervulina. Vaccine 20, 267–274.

Min, W., Lillehoj, H.S., Kim, S., Zhu, J.J., Beard, H., Alkharouf, N., Matthews, B.F., 2003. Profiling local gene expression changes associated with Eimeria maxima and Eimeria acervulina using cDNA microarray. Appl. Microbiol. Biotechnol. 62, 392–399.

Muller, P.Y., Janovjak, H., Miserez, A.R., Dobbie, Z., 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32, 1372–1379.

Pappu, B.P., Angkasekwinai, P., Dong, C., 2008. Regulatory mechanisms of helper T cell differentiation: new lessons learned from interleukin 17 family cytokines. Pharmacol. Ther. 117, 374–384.

Park, J.M., Lee, S.H., Kim, J.O., Park, H.J., Park, J.B., Sin, J.I., 2004. In vitro and in vivo effects of extracts of Lentinus edodes on tumor growth in a human papilloma virus 16 oncogene-transformed animal tumor model – apoptosis-mediated tumor cell growth inhibition. Kor. J. Food Sci. Technol. 36, 141–146.

Park, S.S., Lillehoj, H.S., Hong, Y.H., Lee, S.H., 2007. Functional characterization of tumor necrosis factor superfamily 15 (TNFSF15) induced by lipopolysaccharides and Eimeria infection. Dev. Comp. Immunol. 31, 934–944.

Plattner, F., Yarovinsky, F., Romero, S., Didry, D., Carlier, M.F., Sher, A., Soldati-Favre, D., 2008. Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. Cell Host Microbe 3, 77–87.

Pogonka, T., Klotz, C., Kovacs, F., Lucius, R., 2003. A single dose of recombinant Salmonella typhimurium induces specific humoral immune responses against heterologous Eimeria tenella antigens in chicken. Int. J. Parasitol. 33, 81–88.

Policegoudra, R.S., Abiraj, K., Gowda, D.C., Aradhya, S.M., 2007. Isolation and characterization of antioxidant and antibacterial compound from mango ginger (Curcuma amada Roxb.) rhizome. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 852, 40–48.

Schild, H., Mavaddat, N., Litzenberger, C., Ehrich, E.W., Davis, M.M., Bluestone, J.A., Matis, L., Draper, R.K., Chien, Y.H., 1994. The nature of major histocompatibility complex recognition by gamma delta T cells. Cell 76, 29–37.

Sher, A., Coffman, R.L., 1992. Regulation of immunity to parasites by T cells and Tcell-derived cytokines. Annu. Rev. Immunol. 10, 385–409.

Sodsai, A., Piyachaturawat, P., Sophasan, S., Suksamrarn, A., Vongsakul, M., 2007. Suppression by Curcuma comosa Roxb. of pro-inflammatory cytokine secretion in phorbol-12-myristate-13-acetate stimulated human mononuclear cells. Int. Immunopharmacol. 7, 524–531.

Takimoto, T., Takahashi, K., Sato, K., Akiba, Y., 2005. Molecular cloning and functional characterizations of chicken TL1A. Dev. Comp. Immunol. 29, 895–905.

Trout, J.M., Lillehoj, H.S., 1996. T lymphocyte roles during Eimeria acervulina and Eimeria tenella infections. Vet. Immunol. Immunopathol. 53, 163–172.

Veldhuizen, E.J.A., Tjeerdsma-van Bokhoven, J.L.M., Zweijtzer, C., Burt, S.A., Haagsman, H.P., 2006. Structural requirements for the antimicrobial activity of carvacrol. J. Agric. Food. Chem. 54, 1874–1879.

Wallace, M., Malkovsky, M., Carding, S.R., 1995. Gamma/delta T lymphocytes in viral infections. J. Leukoc. Biol. 58, 277–283.

Williams, R.B., Carlyle, W.W., Bond, D.R., Brown, I.A., 1999. The efficacy and economic benefits of Paracox, a live attenuated anticoccidial vaccine, in commercial trials with standard broiler chickens in the United Kingdom. Int. J. Parasitol. 29, 341–355.

Williams, R.B., Catchpole, J., 2000. A new protocol for a challenge test to assess the efficacy of live anticoccidial vaccines for chickens. Vaccine 18, 1178–1185.

Williams, R.B., 2002. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathol. 31, 317–353.

Yao, Z., Painter, S.L., Fanslow, W.C., Ulrich, D., Macduff, B.M., Spriggs, M.K., Armitage, R.J., 1995. Human IL-17: a novel cytokine derived from T cells. J. Immunol. 155, 5483–5486.

Yarovinsky, F., Zhang, D., Andersen, J.F., Bannenberg, G.L., Serhan, C.N., Hayden, M.S., Hieny, S., Sutterwala, F.S., Flavell, R.A., Ghosh, S., Sher, A., 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science 308, 1626–1629.